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14. ABSTRACT

Blast mediated injuries from Improvised Explosive Devices are strongly associated with severe ocular morbidity and visual impairment and are the leading cause of combat-related injuries. In this study, we documented both *in vivo* microvascular dysfunction in the retina as a function of a blast, but also explored the effects of a therapeutic intervention to halt/reserve the degeneration. To that extent, we use 48 mice split into three cohorts related to how long after the blast (7 days, one month or four months) they were studied and a control group of 16. Our hypothesis is that when arterial flow is pathologically reduced, for example by ocular injury, the flow in the downstream capillary beds is reduced uniformly. We tested this hypothesis with Confocal Laser Endomicroscopy (CLE) *in vivo* imaging to examine the blood flow through the arteriolar vessels and into capillary beds. We also identified mural cells such as pericytes, which have the ultimate active control point for blood flow and regulate non-uniform blood flow in capillary beds. Dysfunctional mural cell-driven non-uniform blood flow can then lead to cell death due to the failure of local oxygenation gradients within the capillary bed. Our hypothesis concerning the mechanistic pathway of action of nitric oxide precursors (i.e. L-Arginine) is that they improve capillary blood flow and prevent ischemia/hypoxia by dilating arterioles and microvessels that would otherwise vasospasm or restrict due to TOI. CLE revealed a significantly different vasospasm rate among cohorts, which are postulated as deriving from mural cell dysfunction in TOI.

15. SUBJECT TERMS

Mural cells, pericytes, vasospasms, traumatic ocular injury, L-Arginine, mice.

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1. INTRODUCTION

The ***objective*** of this research is to determine if clinically available blood flow regulating drugs – currently not purposed to treat retinal damage – may serve to ameliorate retinal degeneration in mice who have experienced blast-related traumatic injuries. We have made progress towards this objective using in vivo imaging techniques along with a mouse model which we developed specifically for blast-type ocular injury. Our ***hypotheses*** are based upon evidence that the vascular effects contribute to neurodegeneration after traumatic ocular injury. We performed experiments to test whether neurodegeneration may be driven by microvascular dysfunction after an overpressure wave. We also performed experiments to test the effects of oral administration of L-Arginine—a nitric oxide (NO) precursor—on ameliorating these vascular contributions. Blood flow control has traditionally been thought to be regulated by arteries, with the last point of active flow control taking place at the pre-capillary sphincters that connect arterioles to primary capillaries. Capillary beds, it follows, irrigate tissue passively: they are driven up and down in their flow uniformly, as a function of their arteriolar supply. The outcome of this model is that when an artery's flow is pathologically occluded—for example, by a stroke or by ocular injury—the flow in the downstream capillary beds is reduced uniformly—as only those neurons very near to microvessels have access to the dwindling oxygen supply—with severe neurodegeneration occurring in watershed areas farthest from vessels. We are now in the final stages of data collection. Which is many months behind schedule due to delayed renovations at SUNY Downstate Medical Center (the Macknik Lab moved to SUNY from Barrow after the decision to award from CDMRP, which introduced delays). We are on-track to complete the data collection of the project in late 2017, and we have a poster accepted for presentation at this year's Society for Neuroscience conference. We will continue to complete the entire project even after the closing of the grant because the grant funds paid for primarily the hardware, and we had always intended to use internal funds for the other costs.

Although the data from each mouse is analyzed shortly after data collection. The evidence to date suggests individual retinal microvessels are affected by TOI, to reduce blood flow control, dur to mural cell (e.g. pericyte) dysfunction, which then contributes to neurodegeneration, presumably due to heightened oxygenation. Our analysis will determine through histological analysis whether neurodegenerating cells will be found predominantly nearby to (spatially associated with) capillary blood vessels, rather than in watershed areas. If this innovative model is correct, it will represent a transformative shift in the field's understanding of cardiovascular function in the eye, to include the capillary beds as the last point of blood flow control, rather than the arterioles.

2. KEYWORDS

Mural cells, pericytes, vasospasms, traumatic ocular injury, L-Arginine, mice.

3. ACCOMPLISHMENTS

- 3.1 What were the major goals and objectives of the project?

The main task to be accomplished in year 1 of this project was to select the ocular trauma model to test the project's hypotheses. This included the establishment of protocols for labelling cells and recording (imaging) techniques, as well as the measurement of baseline function in neurotypical mice. In addition, to conduct fiber-optic imaging to determine blood-flow abnormality and fiber-optic imaging to determine macrophage abnormality.

The main task to be accomplished in year 2 was to record from the retina from WT mice (aim 1), Traumatic Ocular Injury (TOI) mice (aim 2) and TOI mice treated with NO donor L-Arginine (aim 3); and to apply post-hoc analysis to identification arteriolar blood flow abnormalities in normal mice and TOI mice and calculate the contribution of L-Arginine to ameliorating both vasospsams and neurodegeneration.

● 3.2 What was accomplished under these goals?

• 3.2.1 Major activities

First year: setup new dual-band fiber-optic confocal microscope system, test it, and establish protocols for the first successful in vivo retinal microvessel and pericyte advanced microscopic imaging recordings. Identification of first retinal microvessel vasospasms in neurotypical subjects, at rest.

Second year: using the newly established protocol (year 1) we successfully recorded and acquired data from normal mice (aim 1), TOI mice (aim 2) and TOI mice treated with L-arginine (see 3.2.2); in addition, based upon the first imaging results, we assembled the analysis pipeline (analysis protocol, software scripts, etc.).

• 3.2.2 Specific objectives achieved

First year:

- Successful establishment of a protocol to conduct recordings in the retina.
- Successful recordings in neurotypical mice
- Successful recording of normal microvascular vasospasms for the first time.

Second year: as of 9/12/2017 we made significant advances towards completing the recordings for all aims and all cohorts (see table, where the fraction indicates #mice recorded from/ # mice scheduled). We are now waiting for one latest shipment of TOI mice and some mice, housed in our facilities, to age, to finish the recording stage of the project, and subsequently perform the post-hoc analysis and population analysis (expected by the end of 2017).

Cohorts (days after TOI)	7 days	1 month	4 months
Control mice	16/20		
TOI mice	6/10	6/10	0/10
TOI mice treated with L-Arginine	6/10	6/10	0/10

• *3.2.3 Significant Developments, Results and Conclusions*

In year 1, we purchased, set up, tested and calibrated the new dual-band microscope. We experienced difficulties because the pericyte labeling system we used successfully before, did not label the retinal pericytes. Therefore, we modified the protocol by carefully examining all the variables that were changed since we imaged pericytes in the brain. We scrutinized the hardware, software, fluorescent dye protocol, surgical methods, biology (retina vs brain), and even the expertise of the staff, using different tests and methods, which we described in more detail in the annual report following year 1.

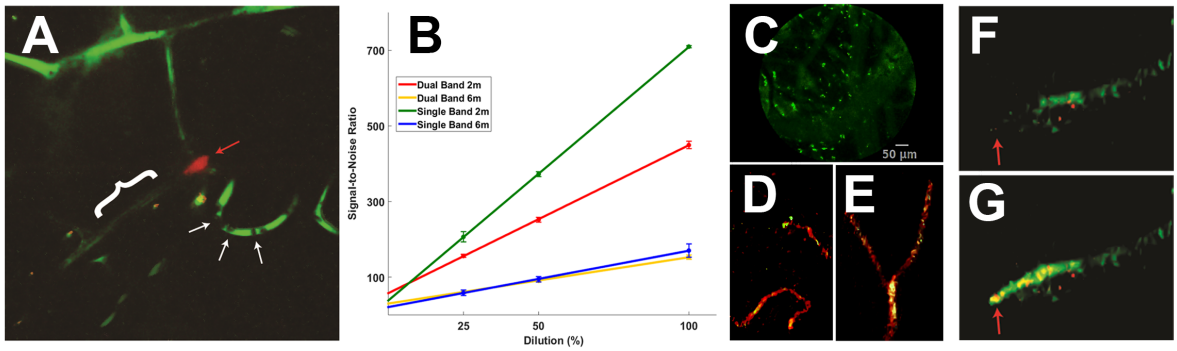

Figure 1: Establish protocol for imaging of retinal blood flow, pericytes and vasospasms in the retinal capillary. **(A)** In vivo labeling of pericytes (red fluorescent dextran, red) in capillaries labelled with 2MD fluorescein dextran in the serum. White arrows point to red blood cells within a capillary. **(B)** SNR as a function of fluorescein dextran dilution for two microscopes (single band vs dual band Cellvizio), and two fiber-optic objectives (2m and 6m). Although we achieve better SNR using a single band, we are required to measure two colors simultaneously and are therefore restricted to use a dual-band. **(C)** The first successful in vivo retinal pericyte recording. **(D)** Image, taken during in vivo fiberoptic recordings in a mouse, showing simultaneous retinal blood flow (red) and pericyte (green). **(E)** Same as (D) but for different mouse. **(F** and **G)** Images of pericyte driven vasospasm in a retinal capillary in a neurotypical mouse. **(F)** Red arrow indicates position of the pericyte but no flow (vasospasm) and **(G)** Red arrow shows same pericyte, now allowing free irrigation of the microvessel.

In short, successfully identified the source of the problem, by using our high quality 2-photon microscope, and found that the retinal mural cells were also labelled by our novel microvascular mural cell staining method, just as we previously showed with brain pericytes (**Fig 1A**); we concluded that our protocol worked well, but that the dyes were too dim to be observed by the new dual-band fiberscope. To address the causes of the signal degradation, we tested the signal-to-noise ratios of fluorescein dextran at different dilutions (dose concentrations) as a function of both our single-band and dual-band scopes, and as a function of fiber length (**Fig 1B**), and as a function of color (red vs green; results not shown). Because of the outcome of those tests, we purchased a set of 2M probes (the ones used for the test were borrowed), and updated our staining protocol, to use green dye (instead of red) for retinal pericytes (**Fig 1C**) and red (instead of green) for blood serum staining (**Fig 1D-E**). By Implementing those changes, we achieved strong retinal pericyte staining with simultaneous blood flow measurements. Following the successful establishment for a protocol to acquire images of pericytes in the retina, we began collecting the vasospasm in healthy mice (**Fig 1F**). The red arrows (**Fig 1F and 1G**) point to a pericyte that is managing the flow of the vessel. When there is no flow in the vessel (**1F**) the pericyte, seen as a small red dot, constricts the flow of blood in the vessel. The fluorescence of that portion of the vessel decreases. As the flow into the vessel increases (**1G**), the pericyte appears to increase in size (this is due to the vessel pressing the pericyte against the face of the fiber when irrigated).

In year 2 we routinely received shipments of mice from our subawardee (Harper Lab) and followed the protocol established in year 1. Because of the delays (see section 5. Changes/Problems), shipments and recordings of mice are still ongoing. As of today, we conducted in vivo experiments using 40 mice (see 3.2.2. for more detail) for this project, and expect to have data collected from 80 mice by the end of this year. Each mouse is prepared by Ms. Nozima (graduate student doing the fiber-optic recordings), who applies retro orbital injection of blood flow dye, anesthetizes the animal and positions the mouse in the fiberoptic microscope; she then records from the retina for 2 to 6 hours.

The recorded images are then passed on to our biomedical engineer, Olivya Caballero, who is blinded to the source of the data and who analyzes the recordings, to select each region of interest (ROI; applied to each frame of the movies by the software), with each ROI containing either a capillary or an artery, as seen in Fig 2, which depicts a capillary that is visible when blood flow is enabled (Fig 2A) but not when the capillary is undergoing a vasospasm (see Fig 2B). Initial analysis reveals that capillaries, but not arteries, regularly experience vasospasms, illustrated by the higher duration (Fig. 2D), greater fraction (Fig. 2E), higher likelihood (Fig. 2F) and higher rate (Fig. 2G)

Population statistics for the results to date are shown in Figure 3. Based upon a the subjects so far, the results suggest the surprising result that vasospasms are less intense (Fig. 3B) and shorter in duration (Fig. 3C) for TOI mice than for normal mice and that L-arginine treatments return the animals to normalcy. In addition, the likelihood that we observe a vasospasm in a given vessel is higher for normal mice than TOI mice (Fig. 3D). Consistent with those results, the fraction of vasospasms per vessel was also greater for the normal mice. These results suggest that blood flow control is reduced after TOI, perhaps leading to increased pathological hyperemia and hyperoxia, which could be the mechanism of neural degeneration after TOI.

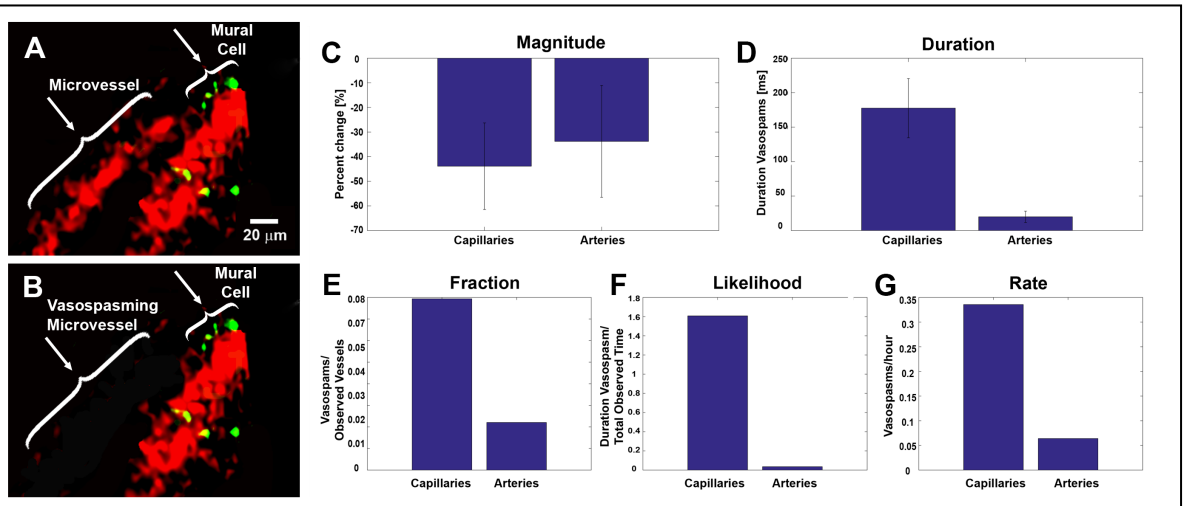

Figure 2: The regions of interest: Arteries and capillaries. **(A)** and **(B)** images showing an artery, branching into a capillary that is controlled by pericytes (green dots; see arrow) and either enables (A) or disables blood flow into the capillary (B). We compared arterial versus capillary vasospasms by measuring: (C) the magnitude of the vasospasm where the error bars represent the SE across all observes vasospasms; (D) the duration of the vasospasm with error bars showing the SE across all measured vasospasms; (E) the fraction of vessels (either capillaries or arteries) experiencing a vasospasm during the time they were observed. (F) the likelihood that a vasospasm occurs when a vessel is observed and (G) the number of vasospasm taking place during one hour of observation.

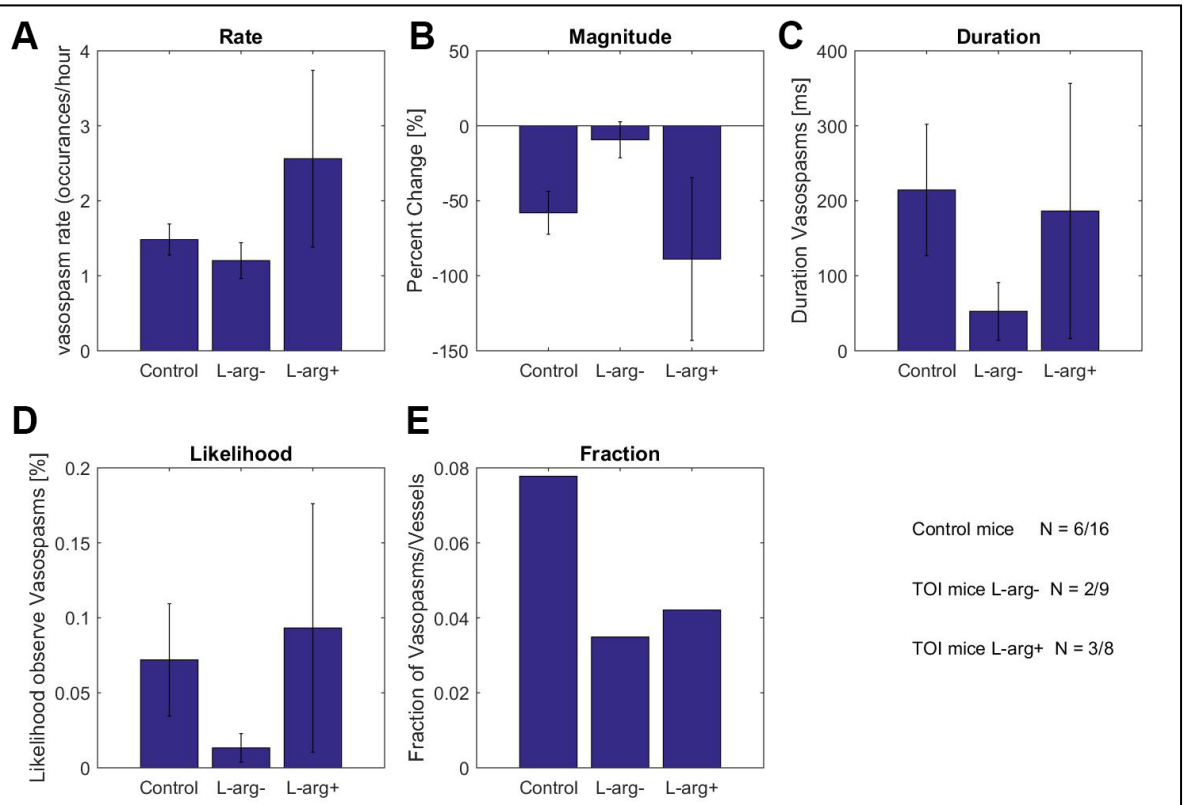

Figure 3: Capillary vasospasms in control mice (Control) and mice exposed to a blast wave to induce traumatic ocular injury (TOI) that were either untreated (L-arg-) or treated with NO donor L-arginine (L-arg+). **(A)** Vasospasm rate, number of vasospasms measured per hour of recording, and averaged across number of mice for each condition. Error bars are +/- SE. **(B)** Vasospasm magnitude, measured as % change in fluorescence, averaged across all mice for a given condition. Error bars are +/- SE. **(C)** The duration of each vasospasm was measured and then averaged per mouse; the results shown in (C) are obtained by averaging across all mice for each given condition and the error bars represent +/- SE. **(D)** Vasospasms likelihood, expressed as a percentage of the total recording time per vessel, averaged across all mice for a given condition. Error bars represent +/- SE. **(E)** Fractions of vessels that were observed and exhibited vasospasms. The results for panel A to D are based upon 6 control mice, 2 untreated TOI mice, and 3 TOI mice treated with L-arginine. Because, for the results shown in panel E, we also took the mice where no vasospasm was observed in account, those numbers were higher: respectively 16, 9 and 8.

The results suggest that the capacity to regulate capillaries is diminished after TOI, resulting in chronic capillary dysfunction. This is different form the results we obtained from our study age-related macular degeneration study (in AMD), where we observed enhanced vasospasms in arteries, which contributed 40% of the neural degeneration in AMD versus age-matched normal mice. Taken together, those studies suggest that the pathomechanic pathways for TOI and AMD are distinct.

● 3.3 What opportunities for training and professional development has the project provided?

As mentioned in our previous report, we trained a graduate student, funded by a different source, who needed to suspend his studies because of mental illness. In year two we trained a first year graduate Student, Adriana Nozima, who conducts most of the mouse recodings, including injection of the dye, recordings from the retina, and imaging of vessels, capillaries and pericytes. Our lab's technician, Manuel Ledo, was also trained to conduct these recordings. In addition, analysis protocols were established by a programming analyst, Jaime Castro, in year 1 and 2, and followed up by a biomedical engineer, Olivia Caballero, in year 2.

● 3.4 How were the results disseminated to communities of interest?

A poster will be presented at the 2017 Society for Neuroscience conference. We expect to submit a manuscript, reporting the results of this project in early 2018.

● 3.5 What do you plan to do during the next reporting period to accomplish the goals?

"Nothing to Report" (final report)

4. IMPACT

● 4.1 What was the impact on the development of the principal discipline(s) of the project?
As reported above (3. Accomplishments), we almost finished with the mice experiments and are in the process of analyzing data. If our model is correct, then we expect that this project will shed new light on our understanding of cardiovascular function in the eye, and the contribution of pericyte physiology to the control of blood flow.
Population statistics will be performed once all data is collected and analyzed; the results might suggest that administration of NO donor L-Arginine ameliorates retinal degeneration caused by traumatic ocular injury, in soldiers. The final results are expected by the end of the current year and might potentially lead to a new line of research in the field of traumatic injury research.

● 4.2 What was the impact on other disciplines?

The data is new and not fully analyzed yet, so there has not been any public reporting of the data.

● 4.3 What was the impact on technology transfer?

"Nothing to Report"

● 4.4 What was the impact on society beyond science and technology?

The data is new and not fully analyzed yet; if the final results indicate that NO donor L-Arginine reduces the number of vasospasms in TOI mice, then this would be an important first step in the identification of a novel treatment with clinical available drug to ameliorate retinal degeneration in soldiers, and civilians, who suffered traumatic injury.

5. CHANGES/PROBLEMS

Due to the PI's move from Barrow to SUNY Downstate at the onset of the project, recordings could not begin until the end of year 1. We also had a graduate student who dropped out, because of health reasons, shortly after he was trained to conduct the experiments associated with this project, and before he was expected to become productive. Despite the setbacks we are now in the process of finishing up the recordings for all three aims and expect to finish the analysis by the end of the current year.

● 5.1 Changes in approach and reasons for change

We didn't encounter any unexpected results which would of have required us to rethink our strategy.

● 5.2 Actual or anticipated problems or delays and actions or plans to resolve them

As reported above we were confronted with some setbacks unrelated to the project; they will not affect the scope, nor the quality or outcome of the project; we reduced the time delay to only a few months and expect to analyze the remaining data by the end of December 2017.

● 5.3 Changes that had a significant impact on expenditures

We had to overcome some staffing problems (see 3.3) and because the people working on this project are funded from a different source, this indeed caused some extra expenditures. In addition, we needed to purchase more mice than initially planned (see 5.4)

● 5.4 Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

We increased the number of mice needed for this experiment, because we needed more mice than expected to establish a protocol for this project (see 3.2.3)

● Significant changes in use or care of human subjects

Not applicable, nothing to report

Significant changes in use or care of vertebrate animals.

Not changes to report

Significant changes in use or biohazards and/or select agents

We tested a number of dyes, and settled for those yielding the best contrast resolution; AngioSEnse 680 EX, and Dextran Fluorescein 10,000 MW, Anionic, Lysine Fixable (Fluoro-Emerald).

6. PRODUCTS

● 6.1 Publications, conference papers, and presentations

- *6.1.1 Journal publications.*
  As reported above we expect to analyze the remaining data by the end of December 2017. Because the preliminary data looks very promising (see 3.2), we are in the process of preparing a manuscript for a high-ranking journal (TBD).
- *6.1.2. Books or other non-periodical, one-time publications.*
  N/A
- *6.1.3 Other publications, conference papers, and presentations.*

The results of this project will be presented during the annual meeting of the Society of Neuroscience in Washington on Monday Nov 13, 2017, during the 8:00 AM – 12 PM session (Session Number 325); under the title: "Microvascular Effects Following Traumatic Ocular Injury"

● 6.2 Website(s) or other Internet site(s)

N/A

● 6.3 Technology or techniques

All methods including protocols to label and image the vasculature as needed for this project and methods to perform 3D-histology will be described in detail when the results of this project will be published and therefore be available to other researchers in our field.

● 6.4 Inventions, patent applications, and/or licenses

N/A

● 6.5 Other Products

N/A

7. PARTICIPANTS & OTHER COLLABORATION ORGANIZATIONS

● 7.1 What individuals have worked on the project?

Name:	**Stephen Macknik**
Project Role:	Principle Investigator
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	6
Contribution to Project	Leading the project; design and troubleshooting; directing and advice team members.
Funding Support	Institutional funds

Name:	**Adriana Nozima**
Project Role:	Graduate Student

Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	12
Contribution to Project	Ms. Nozima prepared the mice, including retro orbital injection, and application of anesthesia; positioning the mice and the fiberoptic microscope; and record from the retina.
Funding Support	Institutional funds

Name:	**Jaime Castro**
Project Role:	Programming analyst.
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	6
Contribution to Project	Mr. Castro established analysis protocols based upon the first imaging results, and then continued analyzing the data.
Funding Support	Institutional funds

Name:	**Olivya Caballero**
Project Role:	Biomedical Engineer
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	3
Contribution to Project	Ms. Caballero took over the responsibilities from Mr. Castro who left our lab in May 2017.
Funding Support	Institutional funds

Name:	**Manuel Ledo**
Project Role:	Lab technician
Research Identifier (e.g. ORCID ID)	
Nearest person month worked:	3
Contribution to Project	Mr. Ledo helped establishing the procedures and surgical techniques during year 1.
Funding Support	Institutional funds

● 7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The PI of this project was awarded an NSF grant during the first half of the current year for an unrelated project; the increase in support did not have a significant impact on the efforts of this project.

● 7.3 What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

● Collaborative Awards:

N/A

● Quad Charts:

The role of each individual who worked on the project, including the funding source, and specific activities, is reported in 7.1

9. APPENCICES

N/A